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[A]Abstract

Species-specific hematological reference values are essential for diagnosis and treatment of disease and maintaining overall health of animals. This information is lacking for many species of elasmobranchs maintained in zoos and aquaria, thus reducing the effectiveness of care for these animals. Descriptive statistics and reference intervals were calculated for hematocrit and complete blood cell counts, biochemistry and protein electrophoresis parameters, trace minerals, vitamins, heavy metals, reproductive hormones, and fatty acids in the blood of 153 wild Sand Tigers *Carcharias taurus* of both sexes and a range of sizes caught in Delaware Bay (Delaware, USA). Mean hematocrit, total white blood cell counts, lymphocyte differentials, glucose, phosphorous, amylase, and aspartate aminotransferase levels were significantly higher in juveniles than in adults. Levels of estradiol, progesterone, testosterone and differences in selenium, and the polyunsaturated fatty acid 20:5n3 (eicosapentaeonic acid) between males and females suggest that they are important parameters for improving Sand Tiger breeding success in managed care. Finally, blood metal levels for arsenic, cadmium, lead, and mercury suggest low levels of contaminant exposure of Sand Tigers in Delaware Bay during their summer residence in the bay. The results of this study provide baseline health parameters for wild Sand Tigers that will aid in effective maintenance of aquarium animals and that contributes to a greater understanding of the biology of these sharks and efforts to accomplish sustainable management of their populations.

[A]Introduction

Species-specific hematological parameters are important tools for evaluating and understanding health and nutrition in both wild and managed populations of animals. Diet, environmental features, activity level, social structure, stress, and parasite loads are all factors that may influence blood values in zoo and aquarium animal collections (Ahmed et al. 2020). Population-based reference intervals for blood analytes are essential for the clinical diagnosis and treatment of disease in fish health, however, this information is lacking for many aquarium species (Otway et al. 2011, Ahmed et al. 2020). Despite the large diversity of elasmobranchs in zoos and aquaria, hematological reference intervals have been established for only a small number of species (e.g., Harms et al. 2002, Cain et al. 2004, Ferreira et al. 2010, Otway et al. 2011, Otway 2015, Cusack et al. 2016), and these results typically have small sample sizes and/or have a limited panel of analytes.

Sand Tigers *Carcharias taurus* are one of the most popular elasmobranch species displayed in public aquaria worldwide (Anderson et al. 2012) due to their large size, hardiness, and impressive dentition. However, aquarium-held Sand Tigers frequently experience health issues that appear to be uncommon in the wild. For example, nearly a third of all Sand Tigers displayed in aquaria have developed spinal deformities, which include spinal curvature, kyphosis, single to multiple incremental subluxations, excessive mineralization of the vertebrae, and/or spinal degeneration (Anderson et al. 2012). Various explanations have been postulated for these spinal deformities, including capture and transport trauma, high growth rates, infection, and nutritional deficiencies (Preziosi et al. 2006, Anderson et al. 2012). Conclusive identification of the causes of spinal deformity have been elusive, partly because of a lack of complementary information for wild Sand Tigers.

Global Sand Tiger stocks have experienced dramatic declines and since they have one of the lowest rates of reproduction among elasmobranchs, full recovery of stocks are expected to require decades (Carlson et al. 2009, Pollard and Smith 2009, Kilfoil et al. 2017). Concern about the negative effects of collecting wild Sand Tigers has also generated keen interest in focused breeding of Sand Tigers in aquaria (Henningsen et al. 2017). Although a variety of reproductive behaviors have been observed in aquarium Sand Tigers (Gordon 1993), successful reproduction under managed care has been extremely limited (Henningsen et al. 2017, Wilson and Smith 2017). This lack of success is likely related to an inability of aquaria to effectively mimic wild environmental conditions, behavioral cues, and/or physiological responses of Sand Tigers to artificial conditions that interfere with reproduction. Understanding the range of hematological values found among wild Sand Tigers is therefore likely to enhance efforts towards successful breeding by providing reference values for wild and aquarium animals.

The objectives of this study were to: 1) provide a comprehensive suite of hematological, biochemical, and nutritional values (complete blood counts, plasma biochemistry, protein electrophoresis, trace minerals, vitamins, heavy metals, hormones, and fatty acids) for wild Sand Tigers in Delaware Bay; 2) compare blood analytes between sexes and among demographic groups of wild individuals; 3) establish reference intervals for wild Sand Tigers for comparison with aquarium individuals; and 4) infer the overall health of individuals collected in Delaware Bay based on hematological parameters.

[A]Methods

[C]*Animal Collection.*—Sand Tigers were caught during August 2011 (N=60) and 2012 (N=93) in Delaware Bay (39°03'00" N, 75°08'59" W) over 15 total days of effort using anchored long lines consisting of 366 m, 0.64 cm braided nylon, with 25 (16/0) circle hooks (barbs depressed) spaced 12 m apart. Hooks were baited with Atlantic Menhaden *Brevoortia tyrannus* or Bluefish *Pomatomus saltatrix*. Longlines were fished during daylight hours using soak times of approximately 2 h. Based on the time required for deployment and retrieval of the longline, however, some individuals could have been on hooks for periods exceeding 2 h. For each long line set, location (determined by GPS), surface water temperature, salinity, and dissolved oxygen were recorded.

Sharks were either immediately brought on deck (< 150 cm total length [TL]) or secured adjacent to the boat with a tail rope (> 150 cm TL). Animals were then rolled into dorsal or lateral recumbency, inducing a natural and temporary state of inactivity (tonic immobility), for workup. Individuals were measured (fork length [FL] and TL), sexed, tagged externally with conventional "spaghetti" ID tags, and a subset received an internal acoustic transmitter for a separate study. Twelve milliliters of blood was collected (<1% of total blood volume) via caudal venipuncture using 18-21 gauge needles of varying length (dependent on animal size) attached to a syringe via an extension set. For sharks processed onboard, the gills were irrigated with saltwater via a hose inserted in the mouth and the skin was kept wet during handling. Total handling time for both onboard and in-water processing was less than 15 min.

[C]*Hematology and Cell Counts.*—Whole blood was immediately transferred into a 1 ml sodium citrate blood tube, a 10 ml lithium heparin blood tube, and an i-STAT™ cartridge for blood gas analysis (see below). Sodium citrate whole blood was added to a cryovial containing a modified Natt-Herrick's stain (Vetlab Supply, Palmetto Bay, FL, USA) at a 1:100 dilution for total white

blood cell (WBC) counts at a later time. Natt-Herrick's stain was modified for elasmobranch osmolality (1,190 mOsm/L) by mixing 25 µl of Natt-Herrick's stain with 0.79 g of urea and 0.34 g of sodium chloride (Arnold 2005; Walsh and Luer 2004). Heparinized whole blood (0.5 ml) was immediately aliquoted for heavy metal testing and held frozen at -80 °C until analyzed. The remainder of the blood was held on ice until centrifuged (1534 x g, 10 min), approximately 60-120 min following collection, and plasma samples were stored frozen at -80 °C until analyzed.

Between 2 and 6 h post-collection, two blood smears were made for a WBC differential count using sodium citrate treated whole blood that had been kept chilled at 4 °C. The blood smears were allowed to air dry and then stained with Diff Quick (Dip Quick Stain Set, Jorgenson Labs, Loveland, CO, USA). Blood smears were examined using light microscopy (1,000× magnification) and the differential count was read twice by identifying 100 individual WBCs and calculating the percentage of each cell type. White blood cells were classified as lymphocytes, neutrophils, basophils, monocytes, coarse eosinophilic granulocytes (CEGs) and fine eosinophilic granulocytes (FEGs) using criteria defined by Arnold (2005). Segmented and nonsegmented CEGs and FEGs were differentiated since they represent differing stages of cell maturity. A manual total WBC count was performed on Natt-Herrick's stained whole blood using a hemacytometer (Walsh and Luer 2004). Hematocrit was measured by microhematocrit centrifugation and total solids were measured by refractometer (HSK-VET, Heska Corporation, Loveland, CO, USA).

[C]*Acid-base and Blood Gases.*— Blood gas analyses were conducted using an i-STAT™ portable clinical analyzer (Abaxis, Union City, CA, USA) and a CG4+ disposable cartridge that measured pH, partial pressure of carbon dioxide (pCO_2, mm Hg), partial pressure of oxygen (pO_2, mm Hg), lactate (mmol/L), bicarbonate (HCO_3, mmol/L), total carbon dioxide (TCO_2,

mmol/L), and percent oxygen saturation (sO_2, %). Since mammalian conversion factors and constants are utilized by the i-STAT to make temperature corrections to pH, pCO_2, and pO_2, water temperature was used as a proxy for body temperature so that values could be of diagnostic use in elasmobranchs (see Mandelman and Skomal 2009).

[C]*Biochemistry Profiles.*—Plasma biochemical profiles were performed at Michigan State University Diagnostic Center for Population and Animal Health (MSU DCPAH) using an Olympus AU640e chemistry analyzer (Olympus Diagnostics, City, NY, USA). This profile included blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, calcium, phosphorous, magnesium, iron, glucose, amylase, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine phosphokinase (CPK), and cholesterol. Reported osmolarity values were calculated based on (2 x Na+) + (glucose/18.0) + (BUN/2.8).

[C]*Protein Electrophoresis.*—Plasma protein fractions were evaluated by an electrophoresis analysis system and gels (SPIFE 3000 system, Split Beta gels, Helene Laboratories Inc., Beaumont, TX, USA) at the University of Miami Avian and Wildlife Laboratory according to manufacturer's instructions and as previously described (Cray et al. 2011). Protein fractions were based on mammalian electrophoresis migration characteristics for albumin, α-1 globulin, α-2 globulin, β globulin, and γ globulin, but identified as fractions 1–5 (Cray et al. 2015). Absolute fraction values (g/dL) were determined by multiplying the percentages for each fraction by total protein (TP) concentrations measured with an Olympus AU640e chemistry analyzer (Olympus Diagnostics, City, NY, USA).

[C]*Reproductive Hormones.*—Plasma hormone levels (estradiol, progesterone, and testosterone) were measured using solid-phase ^{125}I radioimmunoassay (RIA) at Cornell University Animal

Health Diagnostic Center. Prior to being assayed, estradiol samples were extracted with ethyl ether and titrated ^{3}H-Estradiol was used for determining percent recovery of each sample extracted and final calculations. Standard curves were prepared by the laboratory and by Siemen's Coat-A-Count (CAC) Estradiol antibody-coated polypropylene tubes and tracer (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Similarly, for the quantification of progesterone and testosterone, Siemen's CAC reagents were used, which included calibrators with different levels of hormone, antibody-coated polypropylene tubes, and ^{125}I-labeled progesterone or testosterone.

[C]*Trace Minerals and Heavy Metals.*—Trace mineral and heavy metal analysis was based on the methods described by Wahlen et al. (2005). Plasma and whole blood samples were diluted 20-fold with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2% propanol and 20 ppb of scandium, rhodium, indium and bismuth as internal standards. Due to the viscous state after the initial dilution, blood samples were additionally diluted 5x in Millipore water and digested with 3uL ultra-pure 30% hydrogen peroxide for a minimum of 20 min. Whole blood samples were analyzed for arsenic, cadmium, lead, mercury, selenium, and thallium; plasma samples were analyzed for cobalt, copper, manganese, molybdenum, zinc and selenium using an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP/MS, Agilent Technologies Inc., Santa Clara, CA, USA). Elemental concentrations were calibrated using a 4-point linear curve of the analyte-internal standard response ratio.

[C]*Vitamins.*—Plasma vitamin A (retinol) and E (α-tocopherol) levels were analyzed by MSU DCPAH using methods described by Arnaud et al. (1991). Fat-soluble vitamins were extracted with equal volumes of ethanol (which included an internal standard and butylated hydroxytoluene) and hexane. The hexane layer was dried under reduced pressure and dissolved

in chromatographic mobile phase. Samples were analyzed chromatographically using a Waters 2690 Alliance separation module and Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA). Quantification was by internal standard ratios and a six-point calibration curve. Calibration standard solutions were verified by millimolar absorptivity.

Plasma samples were analyzed for water-soluble vitamins (B_3 [niacin], B_5 [pantothenic acid], B_{12} [cyanocobalamin] and C [ascorbic acid]). Plasma vitamin B_3 and B_5 samples were prepared according to methods described by Dawson et al. (1988) and Aslam et al. (2008) and analyzed by high performance liquid chromatography (HPLC) using a Waters HPLC equipped with a 717 plus auto sampler and 510 pump (Waters Corporation, Milford, MA, USA). Plasma samples were diluted for analysis of vitamin C according to methods described by Behrens and Madere (1987) and also analyzed by HPLC. Plasma vitamin B_{12} levels were determined using a competitive binding RIA kit (ICN, Costa Mesa, CA, USA), in which nonspecific vitamin B_{12} binding R-proteins were removed by affinity chromatography.

[C]*Fatty Acids.*—Plasma samples were analyzed for fatty acids as described by Phillips et al. (2010). Samples were methylated as described in Parks and Goins (1994) and reconstituted in hexane prior analysis. Methyl esters were injected into a gas chromatograph (6890 Series II, Hewlett Packard, Avondale, PA, USA) fitted with a flame ionization detector and an automatic injector. Individual fatty acids were identified by comparing their retention times to those of known standards (C12:0 and C27:0) added into each sample prior to methylation (Phillips et al. 2010). Fatty acid results are expressed as weight percentages of total fatty acids.

[C]*Data Analysis.*—Data were tested for normality using Anderson-Darling tests and outliers were identified and removed according to criterion specific to Dixson's and Tukey tests. Patterns

between blood analytes and sex and life-history state (juvenile or adult) were explored using non-parametric Wilcoxon rank sum tests. Correlations between blood analytes, longline soak times, and shark size were explored using linear regression. For life history comparisons, females larger than 220 cm TL and males larger 190 cm TL were considered mature (Goldman et al. 2006).

Reference intervals (RIs) encompass the central 95% of a healthy reference population. In non-normally distributed (skewed) data, percentiles are used to establish reference internals and the 2.5 and 97.5 percentiles are the upper and lower limits for the RIs (Friedrichs et al. 2012). RIs were established for each analyte using nonparametric ranking methods ($N \leq 40$) or by parametric/robust methods from native and Box-Cox transformed values ($N > 40$). Ninety percent confidence intervals (CIs) around these limits were determined non-parametrically ($N > 120$) or through bootstrapping methods ($N < 120$) using Reference Value Advisor freeware (Geffré et al. 2009, Greffé et al. 2011, Friedrichs et al. 2012). Statistical differences in blood analytes between sexes and/or life stage were identified and separate RIs calculated. All individuals included in the determination of RIs were presumed healthy based on physical appearance and external examination. Instances where data were below the analyzer detection limit (e.g., censored data, <8% of total observations) were explored using the Kaplan-Meier estimator as a nonparametric maximum likelihood estimator (Helsel 2006). Median is the only descriptive statistic reported for censored data and RIs were not determined for parameters where more than 50% of the data were censored. Values were considered to be significantly different at $\alpha < 0.05$. Data analyses were performed with JMP (version 10, SAS Institute, Cary, NC) and ProUCL (version 5, ES EPA Research, Durham, NC).

[A]Results

Blood samples were collected from 153 Sand Tigers ranging in size from 122-272 cm TL (101-233 cm FL) over the course of this study. Mean longline soak time was 2.9 ± 0.8 h, with a maximum soak time noted at 5.3 h for single longline set. No differences were detected for any parameter based on year and the data was pooled for subsequent analyses. The sex ratio of captured Sand Tigers was nearly equal (79 females, 74 males) and there was no significant difference in FL ($Z= -0.127$, $p=0.90$) or TL ($Z= -0.20$, $p=0.84$) between sexes. Of the 153 animals captured, 103 were later categorized as juveniles and 53 as adults. Average (± SD) surface water temperature, salinity, and dissolved oxygen were 26.2 ± 1.8 °C, 25.9 ± 2.1 ppt, and 5.3 ± 1.6 mg/L, respectively

[B]Acid Base and Blood Gases

Blood pH was near neutral in all individuals examined, whereas values of pO_2, pCO_2, HCO_3, sO_2, and lactate were highly variable (Table 1). Blood lactate values ranged from 2.3 to 18.1 mmol/L at capture. There were no significant differences between male and female sharks for any of the blood gas analytes (all $p>0.05$). Juvenile sharks had significantly lower sO_2 levels (47.7 %) than adult animals (63.2%, $\chi^2_{1,113}=4.49$, $p=0.0340$, Table 1). Reference intervals were not determined for blood gas analytes since they are impacted by capture and handling and would not represent baseline conditions.

[B]Hematology and Cell Counts

Hematocrit levels did not significantly differ between sexes ($\chi^2_{1,127}=0.282$, $p=0.5952$), but a higher mean percentage of red blood cells were measured in juvenile animals (24%)

compared with adult animals (22.4%, $\chi^2_{1,116}$=9.35, p=0.0022, Table 2). Lymphocytes and CEGs were the most commonly identified WBC, while monocytes and basophils were rarely observed (Table 2). Juvenile animals had significantly higher total WBC (59.7 x 10^3/µl, $\chi^2_{1,51}$=9.96, p=0.0016) and lymphocytes (41.1 x 10^3/µl, $\chi^2_{1,51}$=6.93, p=0.0085), and significantly lower non-segmented FEGs (0.7 x 10^3/µl, $\chi^2_{1,51}$=5.85, p=0.0156), compared with adult sharks (43.0 x 10^3/µl, 22.2 x 10^3/µl, 0.9 x 10^3/µl respectively, Table 2).

[B]Biochemistry and Protein Fractions

Plasma biochemistry values for glucose were high (mean 64 mg/dL, Table 3) compared to values measured in aquarium sand tiger sharks (Anderrson et al. 2012). Values for creatinine were below the level of analyzer detection (<0.2 mg/dL), therefore, reference intervals were not determined. No significant differences were detected between male and female Sand Tigers for any of the plasma biochemistry analytes (all p>0.05). Plasma phosphorous (8.1 mg/dL, $\chi^2_{1,143}$=37.11, p=<0.0001), glucose (66 mg/dL, $\chi^2_{1,143}$=68.27, p=0.009), amylase (831 IU/L, $\chi^2_{1,144}$=14.76, p=0.0001), ALP (18 IU/L, $\chi^2_{1,141}$=36.81, p=<0.0001), and AST (28 IU/L, $\chi^2_{1,143}$=14.31, p=0.0002) levels were significantly higher in juvenile animals compared to adult sharks (6.7 mg/dL, 60 mg/dL, 704 IU/L, 11 IU/L, 21 IU/L, respectively, Table 3).

Plasma protein fraction 1 (0.16 g/dL) and fraction 2 (0.13 g/dL) were low compared to other fractions. The largest globulin fraction was fraction 4 (3.5 g/dL, Table 4). No significant differences in protein fractions were detected between males and females (all p>0.05). Fraction 4 was significantly lower in juveniles (3.4 g/dL, $\chi^2_{1,35}$=7.66, p=0.0057), than in adults (3.8 g/dL, Table 4), while fraction 3 levels were significantly higher in juveniles (1.6 g/dL, $\chi^2_{1,35}$=6.85,

p=0.0088) compared to adults (1.3 g/dL, Table 4). No other differences in protein fractions were detected between juveniles and adults (p>0.05).

[B]Trace Minerals and Heavy Metals

Trace mineral levels in Sand Tigers were similar between sexes for all minerals measured (all p>0.05), with the exception of Se (Table 5). Significantly higher levels of both plasma (203 ng/mL, $\chi^2_{1,155}$=10.68, p=0.0011) and whole blood (746 ng/mL, $\chi^2_{1,154}$=5.34, p=0.0208) Se were noted in males, compared to females (186 ng/mL, 675 ng/mL, respectively). Levels of Co, Mn, Zn and As varied with life history stage (Table 5). Mean Co (2.1 ng/mL, $\chi^2_{1,143}$=9.56, p=0.0020), Mn (15.7 ng/mL, $\chi^2_{1,143}$=6.98, p=0.0082) and Zn (0.48 µg/mL, $\chi^2_{1,143}$=11.44, p=0.0007) levels were significantly higher in juvenile sharks, whereas As levels (1103 ppb, $\chi^2_{1,141}$=28.95, p<0.0001) were significantly higher in adult sharks. A large portion of the analyzed values for Cd (71%), Pb (67%), Hg (77%), and Th (71%) were below the level of analyzer detection (<0.5 ppm, <0.5 ppm, <0.25 ppm, <0.5 ppm respectively) and reference intervals were not determined.

[B]Vitamins

No differences in vitamin levels were detected between male and female Sand Tigers (all p>0.05), however, differences were noted between life history stages for vitamin E (Table 5). Mean vitamin E levels were significantly higher in juveniles (1.19 µg/mL, $\chi^2_{1,143}$=4.93, p=0.0264) compared to adults (1.03 µg/mL).

[B]Reproductive Hormones

Plasma hormone levels varied based on maturity stage and sex (Figure 1). Significantly higher levels of estradiol were measured in females (both juveniles and adults) than in males

(both juveniles and adults) ($\chi^2_{1,97}$=28.22, p=<0.0001), whereas significantly higher levels of both progesterone ($\chi^2_{1,98}$=14.11, p=0.0002) and testosterone ($\chi^2_{1,90}$=44.47, p<0.0001) were observed in males compared to females (Table 6). Juvenile males had significantly lower mean testosterone levels (4.7 ng/mL, $\chi^2_{1,67}$=8.38, p=0.0038) than adult males (6.6 ng/mL). No other hormonal differences were observed between adult and juvenile sharks (all p>0.05). Reference intervals were not determined for plasma hormone levels because hormone values would vary due to the seasonal reproductive cycle of this species.

[B]Fatty Acids

Twenty six individual FAs were detected in Sand Tiger plasma (Table 7). Proportions of FAs in plasma of nearly equal proportions were observed for polyunsaturated FAs (PUFA, m36.0%), saturated FAs (31.5%) and monounsaturated FAs (29.5%). Dominant fatty acids (>5%) in decreasing order of relative importance included: 16:0 (palmitic acid, 17.5%), 18:1n9 (oleic acid, 15.9%), 22:6n3 (docosohexaenoic acid, DHA, 11.7%), 18:0 (stearic acid, 11%), 20:5n3 (eicosopentaenoic acid, EPA, 8.2%), and 20:4n6 (arachidonic acid, AA, 5.9%). The ratio of n-3/n-6 polyunsaturated FAs was 2.5. The only significant difference between sexes was for a single fatty acid, 20:5n3, with male sharks having significantly higher levels of EPA (8.4%, $\chi^2_{1,99}$=5.82, p=0.0159) than females (8.0%). No differences in FAs were detected based on maturity (all p>0.05).

[A]Discussion

This study provides comprehensive hematological reference intervals for a large number of wild Sand Tigers representing both sexes, a range of sizes, and immature and mature and reproductive stages from Delaware Bay. Baseline blood values from wild, healthy Sand Tigers are necessary to inform decisions on veterinary and nutritional health, in order to optimize diet and vitamin supplementation, and to enhance reproductive success in aquarium animals. Blood values can be subject to alteration due to physiological effects of capture and handling-related activities and it is important to view sensitive blood parameters in light of these potentially altering events (e.g., Ahmed et al. 2020). Longline capture of Sand Tigers in this study produced a pronounced physiological stress response resulting in a mixed metabolic and respiratory acidosis (low pH, high lactate, elevated pCO_2). Sharks exposed to acute capture stress typically experience full physiological recovery within 12-24 h of release (e.g., Frick et al. 2012, Kneebone et al. 2013, Kilfoil et al. 2017).

A CBC, including hematocrit, provides information about the types, numbers, and morphology of cells in the blood, which can be used to evaluate animal health and to identify issues such as anemia, dehydration, infection, and trauma. Increases in hematocrit may be induced by a stress event as a result of RBC swelling associated with osmotic upset (Brill et al. 2008). Hematocrit levels (mean 23.5%) measured in this study were comparable to those measured in aquarium Sand Tigers (24-26%) routinely handled for sampling (Stoskopf 2010, Anderson et al. 2012). Total WBC counts in this study (52.3×10^3 cells/µL) were higher compared to counts previously measured in wild Sand Tigers (16×10^3 cells/µL, Stoskopf 2010). Elevated WBC counts are typically indicative of stress, inflammation, parasite burden, and/or disease, but comparison of WBC counts among elasmobranchs is challenging due to inconsistencies in methodology and a lack of standardization in cell type identification.

Knowledge on the functions of elasmobranch white blood cell types is limited and not all elasmobranch granulocytes have a mammalian counterpart (Arnold 2005). Based on the length of time Sand Tigers would have been on a hook in this study and the slow leukocyte response to stress in fishes (e.g., 12-24 h in channel catfish, Davis et al. 2008), it is unlikely that the high total WBC counts we observed would be related to stress of capture. Further, the leukocyte differentials we observed, including a predominance of lymphocytes, were within the normal ranges found in other species of sharks, both in aquariums and the wild (see Arnold 2005, Stoskopf 2010, Haman et al. 2012) An increased granulocyte (neutrophil and/or heterophil depending on the taxa) to lymphocyte ratio in a differential is generally regarded as a better indicator of stress than are total WBC counts (Davis et al. 2008). Granulocytosis (increased neutrophils and heterophils) and lymphopenia (decreased lymphocytes), which can indicate changes in immune strategy (Davis et al. 2008), were not observed in the leukocyte differentials in this study. The most likely explanation for the high WBC counts in this study is that granulated thrombocytes were included in the absolute WBC counts, even though they are not included in the differential. It is unclear if the delayed time to blood processing had any significant effect on total WBC counts. Given the uncertainty surrounding the cause of high total WBC counts, RIs should be viewed with caution.

Standard veterinary biochemical panels typically allow for the evaluation of disease across numerous organ systems using well-defined taxa-specific reference ranges. However, little is known about the origin and physiological function in elasmobranchs of many of the biochemical parameters measured in a standard mammalian panel. Plasma biochemistry values in our study were comparable to serum values reported for wild Sand Tigers captured in Australian waters (Otway 2015) and in healthy Sand Tigers in aquaria (Anderson et al. 2012).

Plasma electrolyte levels (Na^+, K^+, Cl^-) in our study, which can be influenced by physiological capture stress in sharks (e.g., Brill et al. 2008, Frick et al. 2009. Frick et al. 2010), were comparable to values reported for both wild and aquarium Sand Tigers (Anderson et al. 2012, Otway 2015). This suggests that different capture techniques among studies resulted in similar minimal impacts on electrolyte balance for Sand Tigers. Osmolarity levels in our study (815 mmol/L) were lower than values reported for wild Sand Tigers (1082 mmol/L, Otway 2015), although it should be noted that analytical methodologies were different between the two studies. Sodium and chloride concentrations contribute most significantly to the ion portion of plasma osmolarity, and in our study slightly lower Cl^- levels likely explain lower osmolarity levels compared to those reported by Otway (2015).

Urea synthesis in elasmobranchs occurs in the liver and the kidneys regulate blood urea levels with about 95% reabsorption occurring within renal tubules (Ballantyne et al. 1997). Mean values for BUN obtained in our study (845 mg/dL) were lower than levels reported by Otway (1056 mg/dL, 2015). Conversely, TP levels measured in Sand Tigers in our study (5.7 g/dL) were higher than levels reported for wild (3.0 g/dL, Otway 2015) and healthy aquarium animals (3.68 g/dL unknown method, Anderson et al. 2012). While direct comparison of total protein values among studies must be made with caution due to potential differences in analytical methods, BUN and TP can be valuable indicators of feeding status in sharks, with increases in TP and decreases in the ratio of BUN:TP associated with increased feeding rates in sharks (Wyatt et al. 2019).

Sand Tigers in our study exhibited higher mean glucose levels (64 mg/dL) than reported for Sand Tigers captured on SCUBA (48.7 mg/dL, Otway 2015) and in aquarium Sand Tigers routinely handled for blood sampling (34 mg/dL, Anderson et al. 2012). Elevated glucose levels

may be related to feeding status, but are more likely indicative of a physiological stress response. Glucose levels may increase under conditions of prolonged stress in elasmobranchs (Skomal and Bernal 2010) and the onset of glucose mobilization appears to occur prior to the accumulation of lactate in the blood (Wells et al. 1986). Given the potential for capture and handling to elevate glucose levels in elasmobranchs, RI's for glucose should also be interpreted with caution.

Protein electrophoresis has been widely used in human and veterinary medicine to evaluate albumin and globulin fractions (α, β, γ globulins) to assess ongoing inflammatory processes like infectious disease, parasitism, trauma, and/or poor nutritional plane (Cray et al. 2015). Given the unique physiology of elasmobranchs, there is interest in applying this technique for the detection of inflammatory response of managed elasmobranchs since classic plasma biochemistry and hematology testing alone may not be optimal for diagnosing disease in these species (Hyatt et al. 2016). In our study, fraction 1 was small (0.16 g/dL) and consistent with negligible levels of albumin previously reported for other elasmobranchs (Metcalf and Gemmell 2005, Cray et al. 2015, Hyatt et al. 2016). Fraction 2, which shares the migration characteristics of α-1 globulins in other taxa, was low (0.13 g/dL), and was the least distinct fraction in this study and others (Cray et al. 2015, Hyatt et al. 2016). It is unknown which proteins comprise Fraction 3 in elasmobranchs, however, this fraction in mammals, birds, and reptiles comprises the α-2 globulins α-2 macroglobulin, haptoglobin, and ceruloplasmin. Fraction 3 in Sand Tigers in this study (1.5 g/dL) was higher than levels measured in cownose rays (0.72 g/dL, Cray et al. 2015), but not as high as levels measured in bonnethead sharks (2.28 g/dL, Hyatt et al. 2016), possibly reflecting species specific differences in lipoprotein characteristics (Hyatt et al. 2016). Fraction 4, similar to β-globulin migrating faction, was identified as the largest protein fraction in Sand Tigers (3.5 g/dL), similar to what has been noted in other elasmobranchs (Cray et al.

2015, Hyatt et al. 2016) and believed to be correlated with very low-density and low-density lipoprotein cholesterol fractions (Cray et al. 2015). Lastly, fraction 5 is thought to represent γ globulins, specifically IgM, the primary immunoglobulin found in elasmobranchs (Cray et al. 2015).

The protein fractions determined for in this study provide novel baseline information for apparently healthy Sand Tigers which can be useful for evaluating the health and disease state of aquarium cohorts. For example, Hyatt et al. (2016) found significant decreases in Fraction 3 and increases in Fraction 5 of clinically abnormal (e.g., bacterial or fungal infections, skin lesions, wound healing) bonnethead sharks compared with healthy individuals, suggesting this diagnostic technique has great promise for detecting immunological stimulation in response to subclinical inflammatory processes. This is significant given the high incidence of spinal trauma and resulting spinal deformity noted in aquarium Sand Tiger populations (Anderson et al. 2012).

Trace minerals and vitamins are micronutrients required for normal growth, reproduction, health, and maintenance of fish metabolism (NRC 2011). Plasma and whole blood trace mineral and vitamin levels measured in our study are the first reported for this species in the wild. These baselines are valuable for assessing the nutritional health of aquarium cohorts since trace mineral and vitamin levels in fish are largely acquired from the diet (Watanabe et al. 1997). Limited mineral (Zn) and vitamin (A, C, E) values have been reported in aquarium Sand Tigers as they relate to the high prevalence of spinal deformity in this species (Anderson et al. 2012). Mean Zn levels measured in wild Sand Tigers in our study (0.46 μg/mL) were markedly lower than levels reported for healthy sharks in aquaria (mean 7.89 μg/mL), and lower than levels in aquarium sharks affected with spinal deformity (mean 4.12 μg/mL). Similarly, vitamin A (69 ng/mL), vitamin C (0.48 mg/dL), and vitamin E (1.15 μg/mL) levels measured in our study were lower

than reported values in healthy (mean 18,750 ng/mL, 0.62 mg/dL, 7.89 µg/mL, respectively) and affected aquarium animals (mean 14,070 ng/mL, 0.41 mg/dL, 4.12 µg/mL, respectively, Anderson et al. 2012). The vitamin and mineral differences between wild and aquarium Sand Tigers are largely attributed to higher levels in aquarium animals due to vitamin supplementation of the diet, and this is particularly evident in the vitamin A and vitamin E levels reported for managed Sand Tigers. The lower circulating levels of these vitamins and minerals in wild Sand Tigers suggests that nutrition may play a lesser and/or secondary role (to capture- and transport-induced trauma) in the spinal deformity seen in managed populations (Anderson et al. 2012). Additionally, data from this study provides baseline information on circulating vitamin levels necessary for evaluating vitamin supplementation in aquarium populations where over- and under-supplementation of fat-soluble vitamins can be an issue.

[B]Variation with Maturity

Differences in blood analytes were detected with Sand Tiger maturity status (based on total length). Notably, higher values for hematocrit, total WBC counts, lymphocytes, and glucose levels were measured in juvenile sharks compared with adults. Juvenile teleosts have been shown to have both higher total leukocyte and lymphocyte counts compared to adults, which may reflect an immune system that is not fully developed (Ahmed et al. 2020). However, taken together, these elevated values may also indicate that juveniles are more prone to the effects of capture stress than adult Sand Tigers.

We also observed differences in a number of plasma biochemistry values (P, amylase, AST, ALP) between juvenile and adult Sand Tigers in our study. Otway (2015) recorded no variation in serum biochemistry analytes among Sand Tigers of different sizes, ages, and sexes,

with the exception of increased ALP levels with length and age in female sharks, although sample sizes in these sharks were considerably smaller (N=30) than for our study. The significance of variable plasma biochemistry values in our study are not clear, as variations in diet and age may influence P levels (NRC 2011). Amylase is a starch digesting enzyme, and while fish have the same carbohydrate metabolic pathways as mammals, the value of monitoring plasma amylase for the purposes of detecting digestive disorders in elasmobranchs is unknown. Similarly, enzymes traditionally considered useful in serum and plasma biochemistry panels for evaluating potential organ damage in mammals (e.g., ALP, ALT, AST, CK) are difficult to interpret for elasmobranchs given the lack of information about functional significance and tissue specificity of each enzyme (Anderson et al. 2010, Clarke et al. 2012). In mammals, AST is found in all tissues (except bone), with the highest concentrations in liver and skeletal muscle. However, in Red Lionfish *Pterois volitans*, AST enzyme activity is primarily detected in liver and heart tissue (Anderson et al. 2010), suggesting differences in diagnostic significance of plasma AST levels across taxa. Further research on the diagnostic significance of these biochemistry parameters in elasmobranchs is needed. Differences between juvenile and adult Sand Tigers were detected for both trace minerals (cobalt, manganese, and zinc) and vitamin E. The slight differences observed among Sand Tigers in our study may reflect differences in feeding state (fed versus fasted), diet, and/or environment.

[B]Application to Breeding Programs

Differences in blood analytes between sexes may offer insight into physiological processes related to reproduction. Sand Tigers have among the lowest reproductive rate known for sharks (two pups every two years, Gilmore et al. 1983). This aplacental viviparous species is unique in that larger embryos feed on smaller embryos (intra-uterine cannibalism) and then grow

by feeding on unfertilized oocytes supplied by the mother, resulting in a single embryo born per uterine horn (Gilmore et al. 1983, Lucifora et al. 2002). Despite the large numbers of Sand Tigers in aquaria worldwide, successful reproduction has only occurred at four institutions to date (Henningsen et al. 2015).

Estradiol, progesterone, and testosterone levels have not been previously measured in wild Sand Tigers and most of what is known about the reproductive endocrinology of Sand Tigers comes from animals maintained in aquaria. Estradiol concentrations in immature (mean 478 pg/ml) and mature (mean 777 pg/ml) female Sand Tigers in this study were similar to levels measured in serially sampled aquarium females identified as immature (<700 pg/ml) and mature (600-2000 pg/ml, Rasmussen and Murru 1992). Lack of reproductive hormone profile differences between mature animals suggests they were not reproductively active during the month of August when samples were collected in this study. Reproductive hormone cycles of male and female aquarium Sand Tigers suggests that summer months represent a lull in reproductive activity where the lowest levels of steroid hormones were measured with the least amount of variation and low motility was detected in sperm (Henningsen et al. 2008, Wyffels et al. 2020). This also suggests that temporal patterns of the reproductive cycle may be conserved in aquaria. While fisheries data provide some information on the seasonality of reproduction in Sand Tigers in the NW Atlantic (e.g., Gilmore et al. 1983), research focusing on hormonal blood sampling in conjunction with reproductive ultrasound evaluation throughout the year is needed to understand the complete reproductive cycle and timing in this species.

Among the trace minerals, only Se was different between sexes, with male Sand Tigers having significantly higher plasma (203 ng/mL) and whole blood (746 ng/mL) levels of Se compared with females (186 ng/mL and 675 ng/mL, respectively). Selenium is an essential trace

mineral and an integral component of glutathione peroxidase which, in conjunction with vitamin E, protects cells against oxidative damage and prevents nutritional muscular myopathy. As a result, Se compounds are capable of protecting tissues from the toxicity of heavy metals such as Cd and Hg (Watanabe et al. 1997). Dietary Se also plays a vital role in mammalian male reproduction. Male reproductive organ morphology, spermatogenesis, semen quality and motility, and fertility have been shown to be affected by excesses or deficiencies of Se in mammals (reviewed by Ahsan et al. 2014). Persky et al. (2012) also noted significantly higher Se levels in aquarium male Dusky Smoothounds (347 ng/mL) compared with females (230 ng/mL), although at lower circulating levels than measured in this study. The role of Se in fish reproduction is less well understood and limited to work with teleosts (e.g., Ogle and Knight 1989, Penglase et al. 2014). Further research is needed to understand the potential role of Se in shark reproduction, especially considering the lack of successful breeding of managed Sand Tigers.

Fatty acid monitoring is important for animal health since fishes lack the *de novo* ability to biosynthesize long chain polyunsaturated FAs (PUFAs), thus relying on their diet to obtain these essential FAs (Sargent et al. 1999, Trocher 2003). Deficiencies in essential fatty acids can result in fish pathologies including reduced growth, myocarditis, fatty liver, fin erosions, lordosis, reduced reproductive potential, and mortality (NRC 2011). More specifically, dietary fatty acids have proven to be very important in the reproduction of several teleost fish species since they determine gonad composition, affecting not only sperm and egg quality and maturation (Izquierdo et al. 2001, Rodriguez-Barreto et al. 2014, Zupa et al. 2017), but also in the synthesis of eicosanoids that are autocrine mediators in the reproductive process (Sorbera et al. 2001, Tocher 2003).

The individual fatty acids comprising the bulk of the plasma FAs in Sand Tigers in our study included palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1n9), which are preferentially oxidized in fishes (Tocher 2003). Differences in a single fatty acid (20:5n3, EPA) were detected between male and female Sand Tigers in our study. Although it is not known if there are differences in the incorporation of n-3 fatty acids DHA and EPA into plasma fractions between sexes in elasmobranchs, such differences between sexes have been documented in mammals. Males and females differ in their ability to synthesize EPA and DHA from their fatty acid precursor alpha-linolenic acid and this has been linked with circulating levels of sex hormones (Huang and Horrobin 1987, Childs et al. 2008, Walker et al. 2014). In teleosts, the C20 PUFAs play an important structural role in sperm phospholipids and in ovarian physiology. Fatty acids 20:4n6 and 20:5n3 are precursors of eicosanoids, among which, prostaglandins play an important role in vertebrate reproductive function (Stacey and Goetz 1982, Sorbera et al. 2001). The FA composition of fish tissue reflects dietary lipid to a great extent (Henderson and Tocher 1987, Sargent et al. 1989), thus making it easy to manipulate the FA composition of key reproductive tissues by altering dietary inputs. This has direct application to enhancing the reproductive efforts of Sand Tigers in aquaria by comparing the FA composition of wild and aquarium-reared sharks, similar to what has previously been studied in the aquaculture of commercially important teleost species (e.g., Zupa et al. 2017).

[B]Health of Delaware Bay

Sand Tigers can be predictably found in the waters of Delaware Bay during the summer months (June-August), before migrating south (Teter et al. 2015). Inorganic elements in fish are required in trace quantities for normal physiological function (NRC 2011). However, tissue concentrations can also be an indication of the level of environmental pollution, with tissue

levels being influenced by both abiotic and biotic factors, and diet being the primary route of exposure. Trace mineral levels in fish are typically measured in liver and/or muscle tissue as a long-term indication of exposure, while blood values typically depict short-term exposure. Whole blood levels of Cd (<5 ppb), Pb (<5 ppb), and mercury (Hg, <25 ppb) were low in Sand Tigers in our study compared to levels of Cd, Pb, and Hg reported in Spiny Dogfish *Squalus acanthias* (200 ppb, 900 ppb, and 300 ppb, respectively) from the North Pacific (Haman et al. 2012). Conversely, mean blood As levels in Sand Tigers in this study from Delaware Bay (740 ppb) were lower than levels detected in Atlantic Sharpnose *Rhizoprionodon terraenovae*, and Bonnetheads *Sphyrna tibura* (3100-3500 ppb) sampled off the Georgia coast (Haman et al. 2012). While differences in blood fraction, foraging ecology, and environmental exposure make direct comparisons difficult, whole blood metal values measured in this study provide a snapshot of potential low environmental exposure for this migratory species during their time in Delaware Bay.

[B]Conclusions

This study provides RIs for a comprehensive suite of blood analytes for a range of age and size classes of both male and female wild Sand Tigers. This information will improve the evaluation of nutritional status and overall health of Sand Tigers held in aquariums, especially with respect to the common problem of spinal deformity in this population of animals. Values obtained in our study provide a reference for other populations of wild Sand Tigers, some of which have experienced drastic declines or are extremely vulnerable to fishing pressure (Carlson et al. 2009, Pollard and Smith 2009). Additionally, the wide range of parameters obtained for wild Sand Tigers in our study greatly advances understanding of the biology of these animals and provides information likely to improve success of breeding of Sand Tigers in aquaria. Values

obtained for sexually mature, gestating females in future studies would be especially beneficial for enhanced efforts towards breeding of Sand Tigers.

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Table 1. Descriptive statistics for post-capture blood gas parameters (temperature corrected) for Sand Tigers captured by longline.

Parameter	Mean	SD	Median	Min-Max	n
pH	7.2	0.1	7.2	7.0-7.4	124
pO2, mm Hg	30	28	20	5-138	124
pCO2, mm Hg	8.5	1.9	8.8	5.4-12.5	124
HCO3, mmol/L	3.9	0.9	3.9	1.8-5.7	124
sO2, %	50	37	48	3-99	124
Juveniles	48	39	43	3-99	78
Adults	63	30	67	7-99	46
Lactate, mmol/L	8.8	3.3	8.5	2.3-18.1	124

Table 2. Descriptive statistics and reference intervals (RI) with 90% confidence intervals (CI) for plasma total solids and hematological cell counts f Sand Tigers. Descriptive statistics for analyte levels that were statistically significant between males and females or between juveniles and adults are presented under the associated parameter heading. Leukocyte differentials are presented both in absolute and relative form.

Parameter	Diff % (±SD)	Mean	SD	Median	Min-Max	LCI	RI	UCI	n	Distribution	Method
Total solids, g/dL		6.6	0.8	6.4	5.2-10.2	5.2-5.6	5.3-8.2	7.8-10.2	129	G	P
Hematocrit, %		23.5	2.4	24	18-30	18-20	18.2-28.0	27-30	127	NG	RT
Juveniles		24	2.2	24	18-30	18-20	19.3-29.5	27.8-30	89	NG	RT
Adults		22.4	2.5	22	18-27	16.5-18.8	17.5-28	26.4-29.5	28	NG	RT
total WBC, $x10^3/\mu l$		52.9	17.8	52.3	15.5-92.6	15.4-23.8	16.5-90.7	84.2-92.6	53	G	PT
Juveniles		59.7	15.0	57.0	39.8-92.6	33.6-39.7	36-97.6	85.6-110.5	32	G	PT
Adults		43	16.7	40.5	15.5-72.4	ND	9.2-81.8	ND	19	G	PT
sCEG, $x10^3/\mu l$	11.5 ± 5.0	6.1	2.6	5.8	1.1-14.3	1.1-2.6	1.2-13.4	11.0-14.3	53	G	PT
nsCEG, $x10^3/\mu l$	3.5 ± 3.2	1.9	1.7	1.6	0-7.9	0	0-7.8	4.9-7.9	53	NG	RT
sFEG, $x10^3/\mu l$	4.5 ± 3.9	2.4	2.1	2.1	0-8.5	0-1.6	0-8.3	6.3-8.5	53	NG	RT
nsFEG, $x10^{3/}\mu l$	1.6 ± 1.9	0.8	1.0	0.5	0-4.2	0	0-3.7	2.6-4.2	52	NG	RT
Juveniles		0.7	1.0	0.3	0-4.2	ND	ND	ND	32	NG	
Adults		0.9	0.9	0.9	0-3.4	ND	ND	ND	21	NG	
Neutrophils, $x10^3/\mu l$	11.1 ± 4.3	5.9	2.2	5.8	1.6-11.6	1.6-2.3	1.6-11.3	9.5-11.6	52	G	PT
Lymphocytes, $x10^3/\mu l$	66.5 ± 9.4	35.2	5	35.7	25.3-47.1	25.4-28.2	25.8-45.9	42.8-47.1	52	G	PT
Juveniles		41.1	5.4	42.7	28.6-49.0	21.0-32.1	26.7-50.7	48.8-52.1	32	G	PT
Adults		27.2	3.8	26.7	22.8-38.3	20.9-22.9	21.7-39.3	33.6-47.8	21	G	PT
Monocytes, $x10^3/\mu l$	1.2 ±1.2	0.6	0.6	0.5	0-2.6	0	0-4.7	2.0-2.6	52	NG	RT
Basophils, $x10^3/\mu l$	0.2 ± 0.5	0.1	0.3	0	0-1.6	0	0-2.3	0.5-1.6	52	NG	RT

WBC=white blood cell, CEG=coarse eosinophilic granulocyte, FEG=fine eosinophilic granulocyte, s=segmented, ns=non-segmented, ND=not deter LCI=lower 90% confidence limit, UCI=upper 90% confidence limit, G=Gaussian, NG=non-Gaussian, P=parametric method, PT=parametric method Cox transformation, RT=robust method after Box-Cox transformation

Table 3. Descriptive statistics and reference interval (RI) with 90% confidence interval (CI) for plasma biochemistry values in wild Sand Tigers. Values were calculated separately for groups that were significantly different. Descriptive statistics for analyte levels that were statistically significant between males and females or between juveniles and adults are also presented under the associated parameter heading.

Parameter	Mean	SD	Median	Min-Max	LCI	RI	UCI	n	Distribution	Method
BUN, mg/dL	845	123	843	44-1138	44-713	626-1018	1000-1138	153	NG	RT
Creatinine, mg/dL	ND	ND	0.2	ND	ND	ND	ND	153	NG	
Na, mmol/L	254	16	255	200-294	200-224	212-283	278-294	152	NG	RT
K, mmol/L	4.2	0.9	4.3	1.2-5.7	1.2-2.4	2.1-5.5	5.3-5.7	152	NG	RT
Cl, mmol/L	230	14	232	172-258	172-200	185-256	252-258	152	NG	RT
Osmolarity, mOs/L	815	61	813	462-936	462-721	676-913	900-936	152	NG	RT
Ca, mg/dL	15.1	0.8	15.1	12.4-17.1	12.4-13.6	13.6-16.7	16.4-17.1	153	G	P
P, mg/dL	7.7	1.3	7.8	4.2-12.2	4.2-5.5	5.2-10.4	9.7-12.2	152	G	P
Juveniles	8.1	1.1	8.1	4.5-12.2	4.5-6.5	6.1-10.5	9.7-12.2	103	G	P
Adults	6.7	1.4	6.5	4.2-11.6	4.2-5.3	4.2-11.6	9.5-11.6	40	G	P
Mg, mg/dL	2.9	0.3	2.9	0.4-3.9	0.4-2.6	2.5-3.6	3.3-3.9	152	NG	RT
Glucose, mg/dL	64	12	64	36-97	36-44	40-90	88-97	152	G	P
Juveniles	66	11	65	40-97	40-51	43-93	88-97	103	G	P
Adults	60	13	60	36-90	36-38	36-90	79-90	40	G	P
Amylase, IU/L	796	171	789	41-1422	41-572	523-1160	1069-1422	153	NG	RT
Juveniles	831	167	817	504-1422	504-596	543-1232	1108-1422	103	NG	RT
Adults	704	161	712	41-987	41-552	76-987	925-987	42	NG	RT
ALP, IU/L	16	6	16	5-40	5-7	5.8-32.2	27-40	150	NG	RT
Juveniles	18	6	17	6-40	6-9	7-33	28-40	103	NG	RT
Adults	11	4	9	5-22	3.7-5.3	4.3-22	19-25	39	NG	RT
ALT, IU/L	8.5	4.2	8.0	3-22	3-3	3-17	15-22	120	G	P
AST, IU/L	26	14	22	6-89	6-11	10-77	53-89	151	NG	RT

Juveniles	28	15	24	10-89	10-12	11-81	53-89	102	NG	RT
Adults	21	13	17	6-76	6-10	6.2-74.8	41-76	42	NG	RT
CPK, IU/L	105	144	56	15-832	15-19	16-645	448-832	150	NG	RT
CHOL, mg/dL	55	13	53	23-90	23-37	28-82	80-90	152	G	P

BUN=Blood urea nitrogen, ALP= Alkaline phosphatase; ALT=Alanine aminotransferase; AST=Aspartate aminotransferase, CPK=creatinine phosphokinase, CHOL=cholesterol, ND=not determined, LCI=lower 90% confidence limit, UCI=uper 90% confidence limit, G=Gaussian, NG=non-Gaussian, P=parametric method, PT=parametric method after Box-Cox transformation, RT=robust method after Box-Cox transformation

Table 4. Descriptive statistics and reference interval (RI) with 90% confidence interval (CI) for plasma protein fractions in wild Sand Tigers. Descriptive statistics for analyte levels that were statistically significant between males and females or between juveniles and adults are also presented under the associated parameter heading.

Parameter	Mean	SD	Median	Min-Max	LCI	RI	UCI	n	Distribution	Method
Total protein, g/dL	5.7	0.7	5.7	4.6-8.0	4.5-4.9	4.7-7.4	6.9-8.3	35	G	P
Fraction 1, g/dL	0.16	0.09	0.11	0.05-0.4	0.03-0.05	0.04-0.54	0.31-0.90	35	G	P
Fraction 2, g/dL	0.13	0.06	0.13	0.06-0.24	0.06-0.08	0.06-0.24	0.20-0.27	35	G	P
Fraction 3, g/dL	1.5	0.3	1.5	1.0-2.1	0.8-1.1	0.9-2.1	1.9-2.2	35	G	P
Juveniles	1.6	0.3	1.6	1.7-2.1	1.0-1.3	1.1-2.2	2.0-2.4	20	G	P
Adults	1.3	0.3	1.4	1.0-1.7	ND	0.8-1.9	ND	15	G	P
Fraction 4, g/dL	3.5	0.6	3.5	2.4-5.6	2.4-2.8	2.6-5.1	4.5-5.8	35	G	P
Juveniles	3.4	0.7	3.2	2.4-5.6	2.3-2.7	2.5-5.4	4.1-ND	20	G	P
Adults	3.8	0.4	3.7	2.9-4.8	ND	2.8-4.7	ND	15	G	P
Fraction 5, g/dL	0.39	0.08	0.38	0.28-0.56	0.23-0.28	0.25-0.55	0.51-0.60	35	G	P

ND=not determined, LCI=lower 90% confidence limit, UCI=upper 90% confidence limit, G=Gaussian, NG=non-Gaussian, P=parametric method, PT=parametric method after Box-Cox transformation, RT=robust method after Box-Cox transformation

Table 5. Descriptive statistics and reference interval (RI) with 90% confidence interval (CI) for trace minerals, heavy metals, and vitamins in wild Sand Tigers. Descriptive statistics for analyte levels that were statistically significant between males and females or between juveniles and adults are also presented under the associated parameter heading.

Parameter	Mean	SD	Median	Min-Max	LCI	RI	UCI	n	Distribution	Method
As, ppb (W)	740	494	583	282-3175	282-362	326-2596	1779-3175	154	NG	RT
Juveniles	594	269	541	282-2077	282-348	320-1640	937-2077	102	NG	RT
Adults	1103	699	790	460-3175	460-488	461-3159	2752-3175	41	NG	RT
Cd, ppb (W)	ND	ND	5	ND	ND	ND	ND	154	NG	
Co, ng/mL (P)	1.96	0.64	1.96	0.19-4.28	0.19-1.01	0.67-3.28	3.09-4.28	155	G	P
Juveniles	2.1	0.6	2.1	0.4-3.8	0.4-1.1	0.8-3.4	3.1-3.8	103	G	P
Adults	1.8	0.7	1.7	0.2-4.28	0.2-0.99	0.2-4.2	3.0-4.3	41	G	P
Cu, µg/mL (P)	0.38	0.09	0.38	0.02-0.6	0.02-0.23	0.13-0.51	0.49-0.60	155	G	P
Fe, µg/dL (P)	17	8	15	10-62	10	10-42	31-62	125	G	PT
Pb, ppb (W)	ND	ND	5	ND	ND	ND	ND	154	NG	
Mn, ng/mL (P)	13.8	13.7	9.5	0.4-77.0	0.4-2.1	1.16-53.4	44.2-77.1	155	NG	RT
Juveniles	15.7	15.5	10.6	0.4-77.0	0.4-2.16	0.96-65.1	49.9-77.1	103	NG	RT
Adults	9.0	7.7	6.7	0.8-36.3	0.8-1.73	0.82-35.9	20.9-36.3	41	NG	RT
Hg, ppb (W)	ND	ND	25	ND	ND	ND	ND	154	NG	
Mo, ng/mL (P)	1.9	2.6	1.5	0.2-31	0.2-0.7	0.4-3.5	2.9-31.2	155	NG	RT
Se, ng/mL (P)	194	41	196	37-293	37-127	88-266	259-293	154	NG	RT
Males	203	41	208	67-275	67-127	89-266	259-275	76	NG	RT
Females	186	39	190	37-293	37-129	52-270	254-293	78	NG	RT
Se, ng/mL (W)	709	181	685	217-1660	217-467	433-1087	1061-1660	154	NG	RT
Males	746	205	721	217-1660	217-509	424-1287	1069-1660	75	NG	RT
Females	675	148	673	400-1078	400-460	409-1061	937-1078	79	NG	RT
Th, ppb (W)	ND	ND	5	ND	ND	ND	ND	154	NG	
Zn, µg/mL (P)	0.46	0.11	0.47	0.07-0.76	0.07-0.28	0.22-0.67	0.63-0.76	155	G	P
Juveniles	0.48	0.11	0.48	0.07-0.76	0.07-0.29	0.21-0.69	0.63-0.76	103	G	P

Adults	0.42	0.11	0.41	0.12-0.76	0.12-0.28	0.13-0.76	0.54-0.76	41	G	P
A, ng/mL (P)	69	28	64	22-156	22-35	27-144	131-156	143	NG	RT
B3, µg/L (P)	37	13	36	17-59	17-19	17-59	58-59	99	NG	RT
B5, µg/L (P)	284	73	297	153-404	153-157	155-398	387-404	99	NG	RT
B12, ng/L (P)	73	8	74	58-86	58-60	58-85	84-86	99	NG	RT
C, mg/dL (P)	0.48	0.17	0.48	0.07-0.73	0.07-0.20	0.18-0.73	0.72-0.73	100	NG	RT
E, µg/mL (P)	1.15	0.5	1.13	0.22-2.67	0.22-0.44	0.38-2.29	2.06-2.67	155	G	PT
Juveniles	1.19	0.48	1.16	0.22-2.67	0.22-0.45	0.36-2.26	2.06-2.67	103	G	PT
Adults	1.03	0.56	0.80	0.34-2.51	0.34-0.44	0.34-2.50	1.94-2.51	41	G	PT

P=plasma, W=whole blood, ND=not determined, LCI=lower 90% confidence limit, UCI=upper 90% confidence limit, G=Gaussian, NG=non-Gaussian, P=parametric method, PT=parametric method after Box-Cox transformation, RT=robust method after Box-Cox transformation

Table 6. Descriptive statistics for plasma hormone levels in juvenile and adult wild Sand Tigers.

	Males					Females				
Parameter	Mean	SD	Median	Min-Max	n	Mean	SD	Median	Min-Max	n
Juveniles										
Estradiol, pg/mL	125.7	18.0	125.9	92.9-149.6	18	478.3	336.9	465	92.5-1340.2	56
Progesterone, ng/mL	0.28	0.1	0.29	0.1-0.45	18	0.19	0.11	0.18	0.05-0.65	56
Testosterone, ng/mL	4.7	1.8	4.4	1.8-11.4	40	0.65	0.15	0.66	0.37-0.85	13
Adults										
Estradiol, pg/mL	134.8	38.0	142.4	93.6-168.4	3	777.1	191	858.8	505.8-1000	11
Progesterone, ng/mL	0.29	0.17	0.24	0.15-0.48	3	0.17	0.13	0.11	0.05-0.48	11
Testosterone, ng/mL	6.6	3.0	6.2	2.6-16	28	-	-	-	-	-

Table 7. Descriptive statistics for plasma fatty acids in wild Sand Tigers.

Fatty Acid	Mean	SD	Median	Min-Max	n
C14:0, %	0.17	0.24	0.01	0-0.75	99
C15:0, %	0.81	0.68	0.36	0.26-2.96	99
C16:0, %	17.5	2.80	18.4	13.25-22.42	99
C16:1, %	3.50	0.52	3.5	1.73-4.5	99
C16:1 trans 9, %	0.43	0.05	0.41	0.22-0.52	99
C17:0, %	1.04	0.18	1.0	0.16-1.32	99
C17:1n7, %	0.43	0.06	0.41	0.22-0.61	99
C18:0, %	11.0	1.17	11.1	7.0-15.4	99
C18:1, %	4.86	0.72	4.65	3.75-6.84	99
C18:1n9, %	15.90	2.00	16	8.45-20.2	99
C18:2n6 (LA), %	2.44	0.50	2.2	1.39-4.01	98
C18:3n3 (ALA), %	0.57	0.11	0.52	0.31-0.87	98
C20:0, %	0.90	0.13	0.83	0.44-1.12	93
C20:1n9, %	1.23	0.16	1.14	0.8-1.61	98
C20:1n15, %	0.56	0.03	0.56	0.49-0.6	12
C20:2n6, %	0.44	0.06	0.4	0.24-0.62	92
C20:3n3, %	0.71	0.09	0.68	0.37-0.98	97
C20:3n6, %	0.49	0.06	0.47	0.4-0.69	92
C20:4n6 (AA), %	5.89	0.67	5.64	4.6-7.16	99
C20:5n3 (EPA), %	8.20	0.94	8.34	5.25-12.28	99
Males	8.44	1.00	8.4	6.93-12.28	50
Females	8.00	0.83	8.0	5.25-10.72	49
C22:1n9, %	0.48	0.16	0.5	0.37-0.59	2
C22:3n3, %	0.63	0.08	0.59	0.49-0.78	99
C22:4n6, %	1.26	0.17	1.26	0.66-1.94	98
C24:1n9, %	1.22	0.15	1.16	0.57-1.52	98
C22:5n3, %	3.17	0.38	3.02	1.89-3.92	99
C22:6n3 (DHA), %	11.74	1.93	11.75	4.21-18.7	99
Total SFA, %	31.5				
Total MUFA, %	29.5				
Total PUFA, %	36.0				
n-3 polyenes, %	24.9				
n-6 polyenes, %	10.0				
n-3/n-6, %	2.5				

LA=linoleic acid, ALA=alpha linolenic acid, AA=arachidonic acid, EPA=eicosopentaenoic acid, DHA=docosohexaenoic acid, SFA=saturated fatty acids, MUFA=monounsaturated fatty acids, PUFA=polyunsaturated fatty acids